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Enterocytes: active cells in tolerance to food and microbial antigens in the gut

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Summary

Enterocytes used to be studied particularly in terms of digestion protagonists. However, as the immune functions of the intestinal tract were better understood, it became clear that enterocytes are not mere bystanders concerning the induction of immune tolerance to dietary peptides and gut microbiota. In fact, enterocytes are involved actively in shaping the intestinal immune environment, designed for maintaining a non-belligerent state. This tolerant milieu of the gut immune system is achieved by keeping a balance between suppression and stimulation of the inflammatory responses. Our review presents the current state of knowledge concerning the relationship between enterocytes and immune cells (dendritic cells, lymphocytes), with emphasis on the enterocytes' impact on the mechanisms leading to the induction of oral tolerance.

Keywords: enterocyte, immunoregulation, intestinal immunology, oral tolerance, tolerosomes

Introduction

Enterocytes have a clear role in digestion by ensuring the uptake of ions, water, nutrients, vitamins and absorption of unconjugated bile salts. Only recently, it became evident that enterocytes have a much more diverse activity, involving not only chemical processing of food, but also the induction of immunological tolerance to ingested proteins. We may assert that enterocytes participate in the numerous mechanisms leading to the establishment of oral tolerance. For this purpose, enterocytes co-operate with cells of the intestinal mucosa-associated lymphoid tissue (MALT) in order to maintain a non-reactivity state toward dietary and microbial antigens.

In mice, oral tolerance is a physiological phenomenon which commences around weaning age after the seventh day of postnatal life [1], and completes with the maturation of the intestinal epithelium and formation of fully competent tight junctions between enterocytes [2]. In humans, due to a longer gestation, this process starts earlier. Both neonatal and adult oral tolerance is based on the development of regulatory T cells (T_{reg}) with specificity to a certain antigen [3,4]. In the neonatal period, significant T_{reg} development takes place in the mesenteric lymph nodes (MLN), where T cells arrive in a naive state, by expressing the following molecule combination on their surface: L-selectin (CD62L) and the chemokine receptor CCR7 [5], a combination which directs any naive lymphocyte to secondary lymphoid organs. In order to exercise their functions, T_{regs} recirculate from MLN to the intestinal lamina propria (LP) due to integrin $\alpha4\beta7$ expression [6], whose ligand is MAdCAM-1, a molecule expressed solely in the gut [7]. Integrin $\alpha4\beta7$ and CCR9 expression is induced in naive lymph cells by retinoic acid (RA), produced by intestinal dendritic cells (DCs) or by stromal cells in MLN [8,9]. The regulatory phenotype of naive T cells is also induced by transforming growth factor (TGF)-β, a cytokine produced by DCs, mainly by the $CD103^+$ $\alpha v\beta8^+$ subset of DCs. TGF-β promotes the peripheral expression of forkhead box protein 3 (FoxP3) in naive T cells, thus becoming induced T_{reg} (iT_{reg}) [10].

DCs from MLN are instructed to promote the regulatory phenotype in the encountered naive T cells at the time of antigen uptake in the intestinal mucosa. There are two major cell populations with functions in antigen sampling and processing, in LP: $CX3CR1^+$ mononuclear phagocytes (CX3C chemokine receptor 1 is also known as the fractalkine receptor) and $CD103^+$ (αE integrin) DCs [11]. Although $CX3CR1^+$ phagocytes have several features specific for DCs, there is no evidence for their entry into lymphatics and migration to MLN [12] and, thereupon, for their involvement in T_{reg} induction. Furthermore, it appears that $CX3CR1^+$ cells actually participate in priming T helper type 17 (Th17) inflammatory responses [13] to certain bacterial components, sampled directly from the intestinal lumen

[14]. CD103$^+$ DCs thus remain the most important candidates for the development of T_{regs} in MLN, after antigen sampling and migration from LP. Their activity relies on the production of RA and TGF-β. RA synthesis is catalyzed by retinaldehyde dehydrogenase type (RALDH), an enzyme which is not expressed by CD103$^+$ DCs at the time of their arrival in LP [15]. This leads us to the conclusion that DCs evolve towards a regulatory phenotype after entering the intestinal mucosa. The microenvironment in LP is thus responsible for initiating the chain of events that polarize DCs and, respectively, the phenotype of T cells educated by DCs. Given the importance of the gut environment in the polarization of immune cells, one would expect enterocytes to contribute significantly in shaping this microenvironment. In this study we will present the mechanisms orchestrated by enterocytes, together with DCs, in the development of this nursery for tolerant T cells.

Enterocytes block the access of intact antigens in lamina propria

The digestion of luminal nutrients participates significantly in the degradation of epitopes which could give rise to unwanted immune responses. Digestion processes take place mainly in the small intestine – chemical digestion is completed here before the chyme reaches the large intestine, which produces no digestive enzymes. The small intestine is the site where most of the nutrients are absorbed, whereas electrolytes such as sodium, magnesium and chloride, and vitamins such as vitamin K, are internalized in the colon. However, digestive processes cannot lyse all food proteins to the amino acid level. Small amounts of intact proteins are endocytosed by enterocytes, being subjected afterwards to cleavage within the lysosomal compartment. Intact, antigenic proteins are thus prevented from reaching the LP [16,17]. Tight junctions between the apical pole of enterocytes are another factor that contributes to shielding LP against the intestinal lumen content [18]. These junctions are formed of transmembrane proteins – claudins, occludins and junction-associated molecules, connected to the cytoskeleton by another protein structure, zonula occludens. The tight junctional complexes allow only small molecules, less than 500 Daltons in molecular mass, to cross between cells [19]. These types of small molecules are usually not immunogenic. Tight junctions differ in permeability along the intestine, being more permeable in the large bowel than in the jejunum. They are also sensitive to the immune medium in the intestinal mucosa, manifesting an increased leakiness after prolonged exposure of epithelial cells to high levels of tumour necrosis factor (TNF)-α, interleukin (IL)-13 or low levels of IL-10 [20].

An increased transcytosis of intact proteins was found in animal models of allergic diseases, which supports the importance of the intestinal epithelium as a mechanical barrier [21]. In these animals, epithelial permeability of allergens seems to be mediated by CD23/FcεRII and is antigen-specific, given the involvement of immunoglobulin (Ig)E [22]. CD23 is a molecule normally present on the surface of enterocytes, both in humans and in rodents [23]. A high rate of CD23-mediated IgE transfer from the basal to the apical pole of the enterocyte was found in allergic individuals, followed by intraluminal allergen binding and return of the antigen–antibody complex in LP, with the possibility of mast cell activation [24].

The epithelial barrier protects the internal medium not only from food antigens, but also from bacteria. The distal small bowel, caecum and colon have higher bacterial colonization levels than the proximal regions, reaching 10^{12} colony-forming units per gram of intestinal content in the colon. Sixty per cent of the faecal matter mass in humans is due to bacteria. The small intestine contains lower numbers of commensal bacteria as a result of stomach acid, pancreatic enzymes and motility patterns [25]. Instead, the small intestine contains higher levels of nutrients, available for absorption. The distribution of the immune structures is correlated inversely with the density of luminal bacteria. The small intestine has higher numbers of intraepithelial T cells than the colon; it also harbours lymphoid structures such as Peyer's patches, which are absent in the large intestine. Paneth cells, which produce anti-microbial peptides, are almost confined to the small intestine, being only marginally encountered in the caecum and appendix. The rationale for this differentiated development of the intestinal immune system is probably dictated by the need to maintain a tolerant, mutually beneficial relationship with the commensal microbiota.

As surprising as it may appear, the presence of bacteria in the gut lumen contributes to the integrity of the intestinal epithelial barrier [26]. This is achieved by a series of molecular events induced by the gut microbiocenosis. One event is increased synthesis of pIgR (epithelial polymeric immunoglobulin receptor), which provides the translocation of sIgA (secretory IgA) from LP in the intestinal lumen [27] (Fig. 1). sIgA, a valuable local defence tool, prevents unwanted antigens from adhering to the intestinal mucosa. pIgR-deficient mice that lack sIgA and sIgM exhibit an altered barrier function of the intestinal epithelium, but are also more prone to gaining oral tolerance [28]. This argues for a dual function of a competent intestinal mucosa, ensuring both protection against harmful agents and acceptance of small amounts of certain antigens which induce the development of T_{regs}.

Another event triggered by some species of commensal bacteria is the abrogation of polyubiquitination, necessary for IκB-α degradation [29]. IκB-α is the molecule that controls the activity of nuclear factor (NF)-κB, acting as its suppressor. IκB-α degradation is dependent on both phosphorilation and polyubiquitination. A longer life of IκB-α due to suppressed polyubiquitination will result in reduced proinflammatory activity of NF-κB.

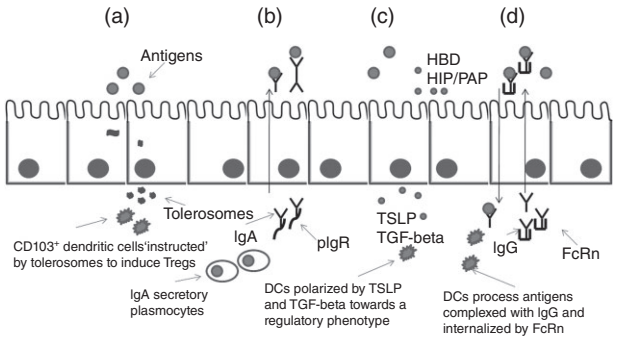

Fig. 1. (a) Enterocytes produce $\alpha v\beta 6^+$ tolerosomes, after processing food or microbial antigens. Apparently, these tolerosomes are directed to $CD103^+$ dendritic cells (DCs). (b) Enterocytes participate in the translocation of secretory immunoglobulin A (sIgA) to the intestinal lumen through epithelial polymeric immunoglobulin receptor (pIgR). In the gut lumen, sIgA is involved in reducing the antigenic pressure exercised on the epithelium, helping to maintain its integrity. (c) Enterocytes produce anti-microbial peptides (AMPs) such as human β-defensin (HBD) and hepatocarcinoma–intestine–pancreas/pancreatic-associated protein (HIP/PAP), which control bacterial development in the close proximity of the mucosa, or cytokines – thymic stromal lymphopoietin (TSLP), transforming growth factor (TGF-β) – to control inflammatory responses. (d) Human enterocytes express FcRn, a receptor which facilitates the recirculation of immunoglobulin (Ig)G complexed with antigens from the gut lumen, mechanism involved in the induction of oral tolerance.

The barrier function of the enterocytes is completed by anti-microbial peptides (AMP) and mucin proteins production [30]. We must specify that AMPs are produced mainly by Paneth cells, and intestinal mucus is the major result of goblet cell activity. Enterocytes produce mucin proteins, which compose the glycocalix, and anti-microbial factors such as β-defensins and hepatocarcinoma–intestine–pancreas/pancreatitis-associated protein (HIP/PAP) [31]. β-defensins bind to the microbial cell membrane and, once embedded, form pore-like membrane defects that allow efflux of ions and nutrients. HIP/PAP is a member of the C-type lectin family and has a promising potential for tissue regeneration and protection against apoptosis and cellular stress, being already tested as an agent for the therapy of acute liver failure in humans [32]. Human β-defensin-1 (HBD-1) is expressed constitutively in enterocytes, while HBD-2 and HBD-3 are induced by microbial products and inflammatory cytokines [33,34]. Inducible expression of HBD-2 and HIP/PAP proteins in enterocytes was shown to be influenced by Toll-like receptor (TLR)- or myeloid differentiation primary response gene 88 (MyD88)-dependent signalling [35,36]. β-defensins may also chemoattract immature DCs [37] and have direct effects on DC function by inducing up-regulation of co-stimulatory molecules and DC maturation [38].

Enterocytes maintain a balance between inflammation and tolerance

Enterocytes possess specialized receptors of the pathogen recognition receptors (PRR) family, such as TLRs and nucleotide oligomerization domain (NOD)-like receptors. These receptors recognize highly conserved molecular structures in bacteria – microbe-associated molecular patterns (MAMPs) – leading to the activation of inflammatory mechanisms which converge to the transcription factor NF-κB. NOD proteins also recognize certain damage-associated molecular patterns (DAMP) of the host cell [39]. Regarding NOD proteins, only NOD1 was found in enterocytes, NOD2 being specific for Paneth cells [40].

Almost all TLRs are present at the mRNA level in enterocytes, but there are differences concerning their distribution along the intestinal tract. By immunohistochemistry and laser capture microdissection of the intestinal epithelium, it was shown that TLR-2 and TLR-4 are expressed at low levels by intestinal epithelial cells (IECs) in normal human colon tissues [41]. TLR-3 is expressed highly in normal human small intestine and colon, whereas TLR-5 predominates in the colon [42]. mRNA coding for all TLR types has been identified in colonic epithelium; the expression of TLR-1, TLR-2, TLR-3, TLR-4, TLR-5 and TLR-9 has also been detected in IECs of the human small intestine [43]. Concerning microbial recognition, TLR-2, -4, -5 and -9 detect bacterial and fungal structures, while TLR-3, -7 and -8 respond to viral products. Signal transmission from TLR to NF-κB is achieved through several adapter proteins, such as MyD88, MyD88 adapter-like (MAL), TNF receptor (TNFR)-associated factor (TRIF) and TRIF-related adaptor molecule (TRAM), which form a complex with the C-terminal domains of different TLRs [44]. NOD1 induces NF-κB activation through receptor interacting protein 2 (RIP2) and a serin/threonin kinase. In enterocytes, TLR and NOD-mediated signalling display specific features which allow the maintenance of minimal proinflammatory cytokine levels, despite increased antigenic pressure from the gut content [31]. Thus, TLR-9 stimulation induces different patterns of protein synthesis. Activation of TLR-9 on the apical pole of enterocytes leads to intracellular accumulation of IκB-α, therefore preventing NF-κB activation, while stimulation of TLR-9 located on the basolateral membrane results in IκB-α degradation. In a similar fashion, enterocytes express TLR-4 only in the Golgi apparatus, unlike macrophages, which express TLR-4 on the plasma membrane. As a result, bacterial lipopolysaccharide present in the gut lumen activates enterocytes only if it penetrates into them [45]. This polarization of enterocytes restrictively enables the initiation of an inflammatory response against microbes that have surpassed the tight junctions between enterocytes and have reached the basolateral membrane; conversely, in contact with the apical region of enterocytes, gut microbes have a limited inflammatory effect [46]. In the same respect of maintaining

tolerance to the intestinal content, enterocytes express a limited number of TLRs in the apical region. Low expression of TLR-4 was observed in colonic biopsies from humans, and low expression of TLR-2 and TLR-4 has been described in human intestinal cell lines [41,47,48]. Furthermore, it appears that, only in enterocytes, TLR-2 stimulation by peptidoglycans leads to activation of the phosphoinositide 3-kinase pathway, which down-regulates NF-κB and promotes barrier integrity and enterocytes rescue from apoptosis [49]. However, TLR activity is a necessity, even at lower rates. TLR-2 or TLR-4 knock-out mice manifest increased susceptibility to colitis after dextran sulphate sodium oral administration [50].

There are also other ways of influencing the NF-κB pathway in enterocytes in order to induce tolerance to MAMPs. For instance, in mature enterocytes, a p50 homodimer form of NF-κB, which lacks the transcription-activating domain, has a higher expression than the proinflammatory heterodimer p50–p65 [51]. In addition, molecules such as IL-1 receptor-associated kinase 4 (IRAK-M), Toll interacting protein (TOLLIP), single immunoglobulin IL-1R-related protein (SIGIRR), zinc finger protein with ubiquitin-modifying activity (A20) and peroxisome proliferator-activated receptor-γ (PPAR-γ) inhibit TLR signalling in human intestinal epithelial cells [52].

TOLLIP ensures a state of non-responsiveness in cultured enterocytes at re-exposure to lipopolysaccharide (LPS), due to down-regulation of TLR surface expression and decreased phosphorylation of IRAK-1 [43]. A20 is a zinc finger protein which inhibits activation of NF-κB via inflammatory cytokine receptors, TLR and NOD2, by ubiquitin-editing activities. A20 suppresses the TLR-2 mediated production of IL-8 in enterocytes and induces hypo-responsiveness to repeated stimulation with LPS [53]. A20 is also an early-response negative regulator of TLR-5 signalling in colonocytes, preventing excessive inflammation after stimulation with flagellin [54].

Another mechanism aimed at maintaining tolerance towards gut content is the mutually exercised inhibition among different inflammation cascades in enterocytes. Enterocytes have two main proinflammatory cascades, mediated by NF-κB and by p38, a mitogen-activated protein kinase [55]. p38 is responsible for synthesis of IL-8, with chemotactic properties [56], and of proinflammatory prostanoids, through cylooxygenase 2 (COX-2) activation [57]. NF-κB activation down-regulates p38, due to NF-κB-induced activation of mitogen-activated protein kinase phosphatase-1 (MKP-1), which dephosphorylates p38 [55].

Enterocytes secrete or respond to regulatory cytokines

An important number of regulatory cytokines were shown to be secreted by enterocytes in response to PRR stimulation. These cytokines directly influence the quality of immune responses primed by LP DCs [58]. Thymic stromal lymphopoietin (TSLP) is a cytokine that activates thymic DCs involved in the positive selection of T_{reg} [59]. TSLP is expressed constitutively by enterocytes and its expression can be enhanced in response to infection, inflammation and tissue injury [60] in an NF-κB-dependent manner [61]. TSLP acts on $CD103^+$ DCs, with subsequent decreased production of the proinflammatory cytokines IL-12 and IL-25, and increased production of IL-10, known for its implication in the development of the Tr1 type of T_{reg} [60,62]. We should point out that TSLP can also activate mast cells [63]. Enterocytes also produce high amounts of TGF-β [64]. This cytokine functions by inhibiting the activity of NF-κB on the promoters of proinflammatory genes in macrophages and DCs [65]. Together with TSLP, TGF-β induces a tolerogenic phenotype in myeloid-derived DCs *in vitro* [66]. TGF-β produced by DCs promotes a Th3 regulatory phenotype in some naive T cells in MLN [67]. TGF-β is also present in human milk [68], and rodent enterocytes have TGF-β receptors [69]. TGF-β is involved in suppressing inflammatory responses in the neonatal gut and in consolidating the barrier function of the intestinal mucosa [70,71]. Enterocytes also influence antibody production in the intestinal mucosa; through TSLP secretion, enterocytes promote B cell activating factor (BAFF) and APRIL (a proliferation inducing ligand) production by adjacent DCs and class-switching of B cells towards the production of sIgA [72,73]. APRIL synthesis is initiated after bacterial stimulation of TLR-4 [74] and results in IgA2 production, an isoform of IgA which is more resistant to proteolysis [75]. After synthesis, sIgA translocates to the intestinal lumen via pIgR; once in the gut lumen, sIgA acts in favour of decreasing the antigenic pressure generated by food and microbes on the mucosa.

Enterocytes display antigens to DCs

Among intraepithelial cells, M cells and enterocytes are capable of mediating the encounter between antigens within the gut lumen and DCs. M cells are dedicated to this function, differing from normal enterocytes which are only secondarily involved in antigen presentation. M cells are located above Peyer's patches (PP) in the small intestine and in close contact with luminal antigens, due to reduced glycocalyx and mucin secretion. They have a particular morphology that allows them to promote uptake and transport of luminal content to professional antigen-presenting cells present in Peyer's patches and lymphoid follicles. M cells possess fewer lysosomes [76], probably indicating a low intracellular antigen degradation, and are present mainly in the small bowel, but also in the colon, rectum or respiratory tract [77]. They are very low in number, counting for only one cell for every 10 million normal enterocytes. Human and mouse M cells express important PRRs, such as TLR-4, platelet-activating factor receptor (PAFR) and α5b1 integrin [78]. These molecules, belonging to the innate immune system,

recognize PAMPs and mediate translocation of bacteria across the epithelium. Jejunal M cells express major histocompatiblity complex (MHC)-II and contain acidic endosomal and prelysosomal structures, indicating that they are able of presenting endocytosed antigens to lymphocytes [79]. It is noteworthy that colonic M cells do not express MHC-II antigens, suggesting that they may not present antigen [80].

Enterocytes are not professional antigen-presenting cells, but they posses MHC-II molecules [81] and produce MHC-II–peptide complexes [82]. This production occurs physiologically at a low rate [83] as part of the immunotolerant mechanisms aimed at counterbalancing an unwanted boost of immune responses. MHC-I and -II expression by enterocytes increases as a consequence of stress and infection. These molecules present antigens to antigen-experienced T cells resident in LP as part of the protective immune response [84].

MHC-II-associated peptides produced by enterocytes can be packed in the form of exosomes, detached from the basal pole. These types of exosomes, in this situation named tolerosomes, participate in the generation of a tolerogenic intestinal environment [85]. The exact structure of tolerosomes is unknown, but it is supposed that they may contain other co-stimulatory molecules, which could induce tolerance to the MHC-associated peptide [86]. The tolerosomes were discovered less than 10 years ago. It has been known from 1983 that oral tolerance is transferrable through serum. Tolerosomes were identified by electron microscopy in 2001, in the serum of animals subjected to induction of oral tolerance, namely in the insoluble fraction resulted by ultracentrifugation. The soluble fraction, containing serum without tolerosomes, could no longer mediate the transfer of oral tolerance [85]. This discovery has proved the existence of intercellular communication through exosomes during induction of oral tolerance.

What exactly happens with tolerosomes after their production is yet not fully elucidated. A recent study suggested that they harbour the αvβ6 integrin and their targets are migratory DCs ($CCR7^+CD103^+$ DC), to whom they convey the necessary information for mounting tolerance to luminal antigens. $CD103^+$ DCs will prime T_{regs} after their arrival in MLN which are specific for the MHC-associated peptide contained in tolerosomes [87]. Another possibility, as an intact portal circulation is needed in order for oral tolerance to develop and tolerosomes are retrievable in serum, could be that tolerosomes are also addressed to DCs in the liver, but this has yet to be proved.

Enterocytes also favour the translocation of intact antigens from the gut lumen into LP. This is achieved in a controlled manner through Ig receptors [88]. In newborn mice, and during the entire human life, neonatal Fc receptor (FcRn) enables internalization of the IgG–antigen complexes [89] as well as IgG externalization, allowing binding to the specific antigen [90]. Most interestingly, FcRn is also present in the mammary glands, where it contributes to exocytosis of IgG–antigen complexes in milk [91]. The excretion of these immune complexes in the human milk induces a state of profound and prolonged oral tolerance in the offspring, due to induction of antigen-specific T_{regs} [92]. FcRn is also found in the placenta, allowing materno–fetal transfer of IgG [93].

The similar transmucosal transportation system of sIgA is better known. It includes the previously mentioned pIgR, as well as a receptor which can re-internalize IgA–antigen complexes from the gut lumen [94]. This second receptor is also expressed by M cells. Antigens complexed with IgA are addressed to DCs from PP, inducing the production of TGF-β and IL-10 [95].

Conclusions

There is growing evidence that the biological process of immune tolerance to food and microbial antigens is not confined solely to lymphocytes; conversely, all the cells in the human intestine play a role in shaping the attitude of the organism towards molecules present in the gut content. Our review emphasizes the participation of enterocytes in this orchestra of mechanisms which preserve the equilibrium between activation and tolerance in the gut mucosa. The ultimate goal of this equilibrium is to decide more clearly when and against which it is necessary to fight back in order to preserve our integrity as an organism. In this context, enterocytes constitute more than a physical barrier against foreign substances from the gut; they are capable of reacting intelligently to the heavy antigenic load of the gastrointestinal tract. Through their diverse array of receptors, antimicrobial peptides and regulatory cytokines, enterocytes are true immune-competent cells. The fineness of the immune mechanisms displayed by enterocytes, in conjunction with the complex design of the local lymphoid tissue, is yet to be elucidated. A better understanding of 'who and how' is responsible for developing oral tolerance will ultimately offer us the tools for manoeuvering in a wide range of clinical situations.

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Disclosure

The authors have no conflicts of interest to declare.

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